PREPARATION OF SUPRAMOLECULAR AMPHIPHILIC CYCLODEXTRIN BILAYER VESICLES FOR PHARMACEUTICAL APPLICATIONS

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Kate E. Frischkorn

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COMMITTEE MEMBERSHIP

TITLE: Preparation of Supramolecular Amphiphilic Cyclodextrin Bilayer Vesicles for Pharmaceutical Applications

AUTHOR: Kate E. Frischkorn

DATE SUBMITTED: June 2018

COMMITTEE CHAIR: Sandra Ward, Ph.D.
Assistant Professor of Chemistry

COMMITTEE MEMBER: Erik Sapper, Ph.D.
Assistant Professor of Chemistry

COMMITTEE MEMBER: David Clague, Ph.D.
Professor of Biomedical Engineering

ABSTRACT

Preparation of Supramolecular Amphiphilic Cyclodextrin Bilayer Vesicles for Pharmaceutical Applications

Kate E. Frischkorn

Recent pharmaceutical developments have investigated using supramolecular nanoparticles in order to increase the bioavailability and solubility of drugs delivered in various methods. Modification of the carbohydrate cyclodextrin increases the ability to encapsulate hydrophobic pharmaceutical molecules by forming a carrier with a hydrophobic core and hydrophilic exterior. Guest molecules are commonly added to these inclusion complexes in order to add stability and further increase targeting abilities of the carriers. One such guest molecule is adamantine combined with a poly(ethylene glycol) chain. Vesicles are formed by hydrating a thin film of amphiphilic cyclodextrin and guest molecules in buffer solution that mimics physiological conditions. The solution is subject to freeze-thaw cycles and extrusion, and the complexes are separated out via size exclusion chromatography. Dynamic Light Scattering instrumentation is used to observe the particle size distribution. Cargo release can be observed in fluorescent dye-loaded vesicles by addition of a membrane-cleaving agent under a fluorimeter instrument. Future work involving this drug delivery technology includes synthesizing a chemically sensitive guest that will cleave in the presence of an intra-cellular anti-oxidant, and finally observing the uptake of these vesicles into live cells and testing the delivery of cargo in vitro under physiological conditions.

ACKNOWLEDGMENTS

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1. INTRODUCTION

1.1 Current Drug Delivery Methods

The pharmaceutical industry is one that is wildly diverse and rapidly expanding due to emerging technologies and techniques. There have been countless drugs and drug-related products since the beginning of mankind, ranging anywhere from ancient holistic remedies to cutting-edge synthetically manufactured medications. The wide range of variability of pharmaceutical products extends to their function as well, treating mild headaches or a multitude of symptoms resulting from a complex disease. As there are so many different kinds of drugs and ailments they help treat, there is also a wide array of delivery methods. These methods are determined by the pharmaceutical products' specific molecular composition, intended target inside the body, and dosage, to name a few factors. Some of the most common drug delivery methods include oral dosage, parenteral, and transdermal.[1]

While these methods of delivery make up a majority of drugs on the market currently, there are several issues that could be improved upon. The main issue with these methods is poor solubility and bioavailability. With about 85% of drugs sold being orally administered (pills, capsules, gels, liquids, etc.), this affects a large majority of the pharmaceutical industry.[2] Less of the product reaches the intended target if it is not readily bioavailable; higher concentrations of the product are required during manufacturing in order to have the intended therapeutic response. Another main issue with oral drugs is the harsh physiological conditions they are exposed to throughout the digestive system.[3] The drug must be able to

withstand harsh pH conditions and the lengthy journey to the small intestine, where absorption occurs. For this reason, peptide or macromolecular drugs cannot be administered orally. Parenteral drugs are able to administer the types of molecules not compatible with oral dosage, but they are not as widely used. Due to the need for trained professionals for administration, a decrease in patient compliance, and higher pain that comes with injected medications, they are often more difficult to produce. Transdermal deliveries offer a simple method of drug administration, but they are not as efficient in systemic deliveries. The pharmaceutical product must be able to pass through several layers of tissue in order to reach the bloodstream and achieve systemic distribution.[3]

There are several methods that are being explored in order to improve the bioavailability of pharmaceutical products. Due to the variation among the drugs' unique chemistry and intended use, there is not a one-size-fits-all type of solution. However, there have been recent advancements in using polymeric chemistry to improve many aspects of the pharmaceutical industry.

1.2 Amphiphilic Polymers for Drug Delivery

Drug delivery systems incorporate both traditional delivery techniques with newly engineered technologies.[4] Polymers, with all their versatility and tailor-ability, are becoming more popular to include in pharmaceutical manufacturing. Advancements in polymer chemistry in recent years have allowed more affordable and efficient experimentation to alter pharmaceutical products.[5] For example, certain polymers can be added to oral dosage drugs in order to improve the taste,

and polymeric coatings can make swallowing easier. Targeting to a specific location in the body can be done by including specific moieties to the formulation. One such example is adding the protein transferrin (Tf) to a delivery system, as its receptor (Tfr) is in higher abundance in tumor cells than healthy ones.[12] This type of targeting ensures that the drug delivery platform has a higher chance of delivering anti-cancer drugs to the tumor site through receptor-mediated endocytosis. Specific targeting in drug delivery systems can include more than just ligand-receptor interactions.

Polymer drug delivery systems can be designed to be highly responsive to the surrounding environment.[6] Depending on the polymers used, delivery platforms can be responsive to either chemical or physical stimuli. Chemical stimuli include pH, ionic strength, or presence of particular molecules. Ester and acetal groups are common functional groups that are pH sensitive, both of which can be cleaved at high pH. Disulfide linkages are readily reduced by certain antioxidants in high intracellular concentrations, such as cysteine or glutathione.[7] These bonds and functional groups can be included in drug delivery polymers as a way to engineer controlled release of an encapsulated therapeutic agent. Figure 1, below, shows examples of several compounds containing disulfide bonds. These can be used to synthesize other polymeric molecules.

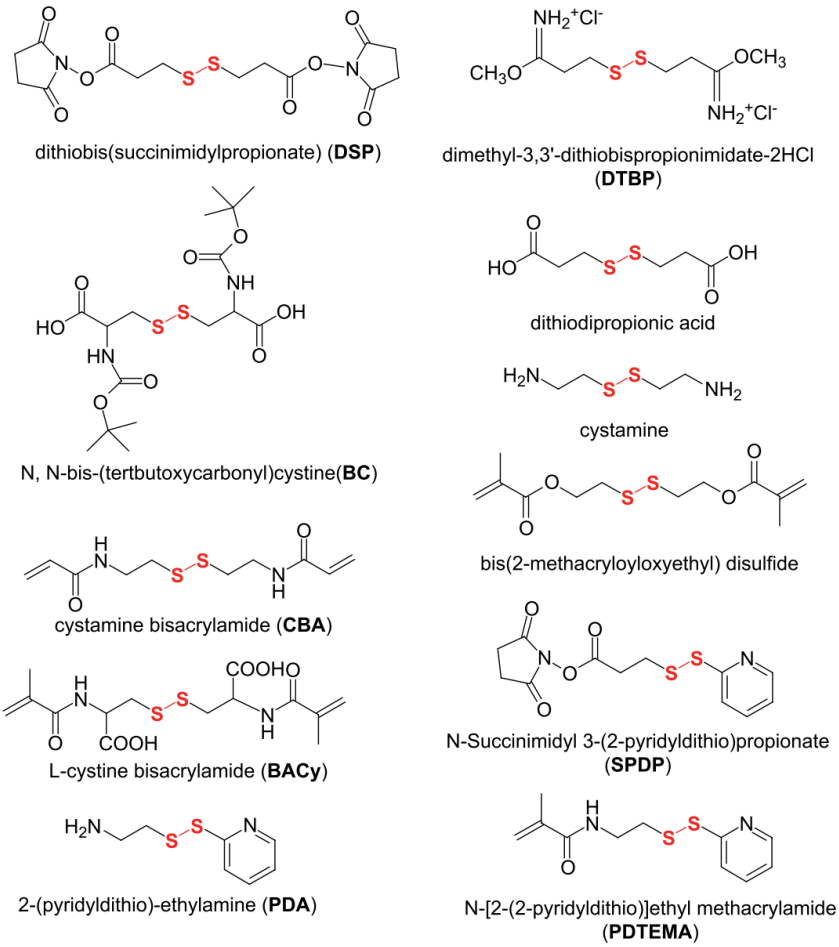


Figure 1. Examples of chemical structures containing disulfide linkages that are redox-sensitive.

Exposures to temperature differences, ultrasound, or magnetic fields are examples of physical stimuli. These physical triggers cause a change in the energy of the polymer system, leading to conformational or solubility changes. An example of a thermos-responsive polymer is PNIPAAm, which takes shape of a hydrophilic coil below 32° C and collapses into a hydrophobic globule at higher temperatures (Figure 2). Understanding the behavioral and chemical responses of polymers

under certain physiological conditions allows for controlled targeting and release of drug molecules.

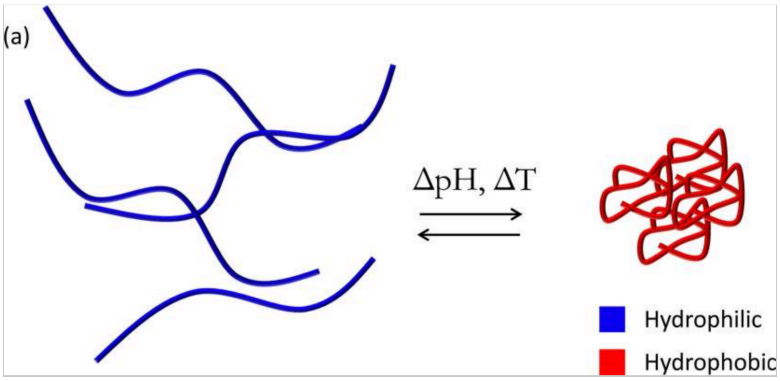


Figure 2. Example of chemically (pH) or physically (temperature) responsive polymer. Addition or removal of stimulus creates a reversible transition between hydrophilic coils and hydrophobic globules.

Another polymeric method of improving efficiency of pharmaceuticals is the use of dendrimers. These polymers are highly branched and spherically shaped.[8] The center of the sphere is the initiating core, with layers of polymers growing outwards in a 3D shape. Drugs can be loaded into the dendrimers in two different ways as seen in Figure 3: either through van der Waals forces holding the drug into the cavity between branches, or by including functionality on the surface that will covalently bind with the drug. The surface layer of the dendrimer can include different functional groups for specific targeting as well.[9] The library of available monomers that can be used to build a dendrimer drug carrier is near limitless, so this method can be easily tailored to a particular drug or application for the desired

delivery. Transdermal and oral dosage drugs are the most common administration route of dendrimer carriers.[10]

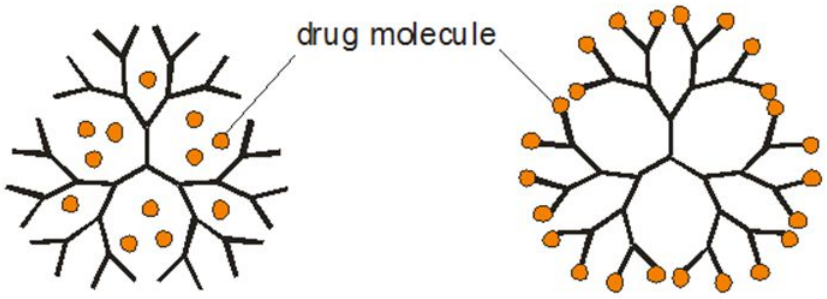


Figure 3. Dendrimers as drug carriers. Drugs can be loaded into the open cavities within the dendrimer (left) or covalently bonded to the surface layer (right).

1.3 Supramolecular Amphiphiles for Drug Delivery

A broader category of drug delivery is supramolecular nanoparticles. These assemblies are in the nano-range, making them ideal for transportation through tissue and cellular membranes.[11] They are composed of several molecules self-assembled and stabilized by non-covalent interactions. In high enough concentrations, they form 3D structures like micelles, bilayers, and vesicles.[13] Hydrophobic and hydrophilic interactions most commonly cause these particular assemblies to form, although electrostatic interactions are common as well. These 3D structures contain inner cavities that are capable of loading small molecules into. Some of these containers can even load up to 20-30 wt.% into their cores.[14]

Lipids are gaining popularity in the drug carrier field due to their low toxicity and ability to encapsulate hydrophobic, hydrophilic, and amphiphilic molecules.

Phospholipids fall in this category and are most well known for making up cellular membranes. This membrane is composed of two layers of phospholipids held together via hydrophobic-hydrophilic interactions. The hydrophilic heads of the lipids face outwards on both sides, while the hydrophobic tails face inwards towards each other. Due to the similar amphiphilic nature of both cell membranes and supramolecular nanoparticles, these assemblies are being investigated as potential drug carriers. Positively charged liposomes (vesicles made from lipids) are being used to carry negatively charged siRNA for gene therapy.[13] The mimicry of the natural chemical structure of the cell membrane allows the nanocarrier to pass through the membrane in order to deliver the drug cargo directly to its intended target. Poly(ethylene glycol) (PEG) chains can easily be grafted onto liposome surfaces to increase water solubility and reduce aggregation. In addition, the hydroxyl groups on the end of the chains can be further modified for targeting or controlled release, such as adding a ligand to target specific proteins.

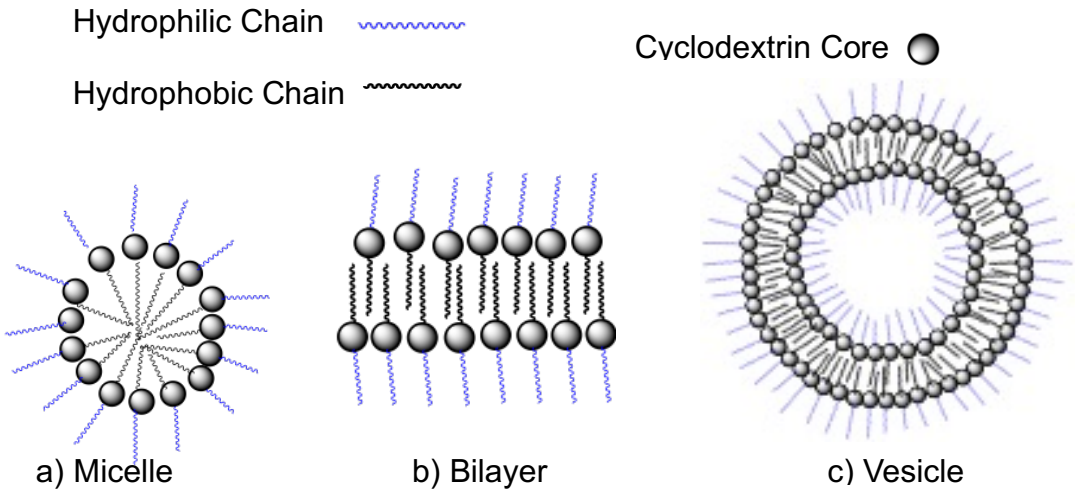


Figure 4. 3D self-assemblies of amphiphilic supramolecular nanoparticles.

Using these lipid systems as inspiration, polymers and other organic molecules can be modified in order to have these hydrophobic-hydrophilic regions to create 3D structures for drug delivery using supramolecular chemistry, shown in Figure 4 above. One of the most used amphiphilic supramolecular nanoparticles used for drug delivery is modified cyclodextrin.[12]

1.4 Amphiphilic Cyclodextrin Vesicles

1.4.1 Cyclodextrins

Cyclodextrins (CDs) are macrocyclic oligosaccharides composed of several simple sugar molecules bonded together in a ring to form a truncated cone (Figure 6).[5] Different types of CDs can be used, most commonly α-, β-, or γ-cyclodextrins (having 6, 7, or 8 sugars, respectively (Figure 5)). The inside of the ring forms a hydrophobic cavity, which is ideal for creating inclusion complexes with other organic molecules, and the outer surface is hydrophilic.

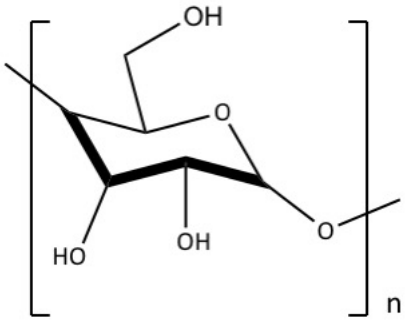


Figure 5. Cyclodextrin, in which n = 6, 7, or 8 for α-, β-, or γ-CD, respectively.

CDs gained popularity over 100 years ago with their discovery by Villiers and Schardinger, but have only recently had potential pharmaceutical applications.[15] This time difference is due to recent advancements in biotechnology that have allowed more cost-efficient mass production and purification of cyclodextrins. With production costs much lower in recent years, the use of CDs in pharmaceutical manufacturing has increased exponentially. These molecules are in part attractive because of their inherently low toxicity levels, as well as how bioavailable they are. The hollow cavity gives the opportunity to load guest molecules through van der Waals forces.[11] This allows for secure encapsulation of either a drug or another type of guest molecule without covalent bonds. CDs are not readily soluble in water, which is why modifications to the hydroxyl groups are necessary to increase water solubility. Modifications that create an amphiphilic structure also allow the CDs to self-assemble into bilayer vesicles. These hollow spheres have an aqueous interior, which can encapsulate small molecules.[16]

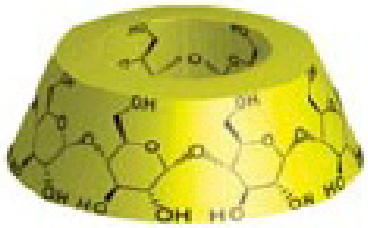


Figure 6. 3D configuration of β-cyclodextrin.

1.4.2 Amphiphilic Cyclodextrins

Bart Jan Ravoo, a professor at Universität Münster, has further progressed the applications of CD vesicles in his work. The modifications that have improved

the potential to form these bilayers involve substituting a hydrophobic alkyl thioether for a hydroxyl group on one side of the cyclodextrin.[17] The α- and β-CDs are better suited for pharmaceutical applications due to the fact that they are not hydrolyzed by proteins in biologic fluids.[15] β-CDs are used more than α-CDs because their cavities are more voluminous and can therefore accommodate larger guest molecules. The surface-to-volume ratio of β-CDs is attractive for individualizing the molecules in order to demonstrate the desired characteristics, such as charge, size, specific functional groups, or targeting proteins.[12] A large library of guest molecules that are compatible with CD cores is increased even more when they are conjugated with other molecules to add additional desired characteristics. Ravoo has made additional modifications to cyclodextrins to increase water solubility: addition of hydrophilic molecule to hydroxyl groups on the side of the CD opposite the hydrophobic thiol chains, as pictured in Figure 7.[17] Hydrophilic poly(ethylene glycol) PEG chains were appended to the secondary side of the CDs, thus making the CD amphiphilic. This increases solubility and self-assembly by sterically hindering CDs from aggregating and allowed neat bilayers to form. Another advantage of using cyclodextrins as drug carriers is relatively easy synthesis. No complicated or expensive reagents or techniques are used in Ravoo's procedures. This benefits pharmaceutical manufacturers by decreasing cost of production and time required to synthesize these drug carriers, thus increasing product output and marketability.

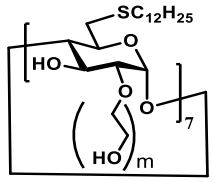


Figure 7. Amphiphilic β-CD modified with hydrophobic thioalkyl chain and hydrophilic PEG chain.

Guest molecules used in inclusion complexes with cyclodextrins are most commonly adamantane (Ad) derivatives due to a high binding affinity.[12] These guests aid in formation and stabilization of the vesicles. The round-shaped, hydrophobic adamantane fits easily into the hydrophobic cavity within the cyclodextrins and is stabilized by conformational changes and van der Waals forces, shown below in Figure 8. Adamantane can be tacked on to a large variety of polymeric molecules, depending on the desired functionality.

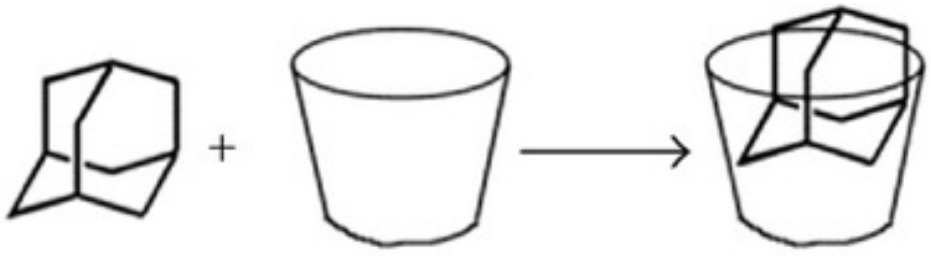


Figure 8. Adamantane (left) and cyclodextrin (middle) forming an inclusion complex (right).

A very common polymer to use for the guest molecule is poly(ethylene glycol) (PEG). This polymer is incredibly versatile, and when bonded with Ad, it makes a guest molecule that increases water solubility and vesicle formation with

modified CDs. The end hydroxyl of the PEG chain can be further modified to tailor to specific requirements. Biomarkers for targeting, radioactive molecules for imaging, and large polymers to increase vesicle size are all examples of further Ad-PEG guests.[12,16] Ravoo creates a redox-responsive polymer shell attached to vesicles formed by amphiphilic CDs through Ad-derivatives that form disulfide bonds.[18] This supramolecular nanoparticle is held together by including an adamantane-derived guest molecule that connects the CD to the crosslinked polymer shell. This shell contains disulfide bonds that are cleaved by glutathione (GSH), an antioxidant naturally found in most living cells. It is a reducing agent that breaks apart disulfide bonds in high enough concentrations, such as inside cells. By using disulfide bonds to stabilize drug-carrying supramolecular nanoparticles, cleavage of the disulfide bond causes the nano-assembly to fall apart and release the cargo encapsulated inside. Intracellular environments have higher concentrations of GSH than extracellular matrices, so these inclusion complexes can target load-release inside cells. Intracellular concentrations of GSH are measured to be around 2-10 mM, while extracellular concentrations are much smaller, at 2-20 μM.[18]

1.4.3 Characterization of Vesicles

Several methods are used to characterize the formation and properties of vesicles.[17,18,19] Scanning electron microscopy (SEM) and transition electron microscopy (TEM) are methods in which to observe the surface structure of a sample. They are similar in that they both use electrons to view, but SEM only scans the surface with a beam of electrons while TEM transmits electrons through

a sample. Dynamic light scattering (DLS) uses lasers shining through a solution to observe the particle size distributions within the sample.[20] There are three different methods to categorize the size distributions: intensity, volume, and number. The intensity distribution measures how intense the reflected light is for each different diameter detected. The volume distribution shows the percentage of total volume taken up by each measured diameter particle. Used for the application of cyclodextrin vesicles, the number intensity is measured, which measures what fraction of the total number of particles a particular diameter is. Ravoo reported an average range of 80-140 nm for vesicles formed by amphiphilic β-cyclodextrins.[17] A simple way to determine successful encapsulation of hydrophilic molecules by CD vesicles is addition of water-soluble carboxyfluorescein (CF) to the buffer solution used to prepare vesicles.[18] The CF is added at a self-quenching concentration, so any dye encapsulated by the vesicles produces low fluorescent measurements. Using a fluorimeter instrument, a small sample of vesicles is added to excess buffer and the fluorescence is continuously measured. During the measurement, a lysing agent can be added to the solution to force degradation of the vesicles. A peak in fluorescence from the released CF, no longer at self-quenching concentration, is indicative of successful encapsulation.

1.5 Project Goals

The following report explores the preparation of bilayer vesicles made from modified amphiphilic cyclodextrin with adamantane-derivative guest molecules. β-cyclodextrin was solely used instead of α- or γ-cyclodextrins, due to the increased

applicability for pharmaceutical delivery. The only modification to these CDs is the addition of an alkyl thioether on the primary side to make these host molecules hydrophobic. Adamantane derived guest molecules were synthesized in order to stabilize the vesicles in aqueous solutions and provide a hydrophilic region to drive bilayer formation. Dynamic light scattering was used to observe the particle size distribution to check for reported vesicle size range. Proper encapsulation was tested for in a fluorimeter by lysing CF dye-loaded vesicles. The overall goal of this project was to create bilayer vesicles using supramolecular chemistry between CD containing hydrophobic chains and a hydrophilic adamantane-derived guest molecule containing a redox-sensitive cleavable disulfide bond, depicted in Figure 9.

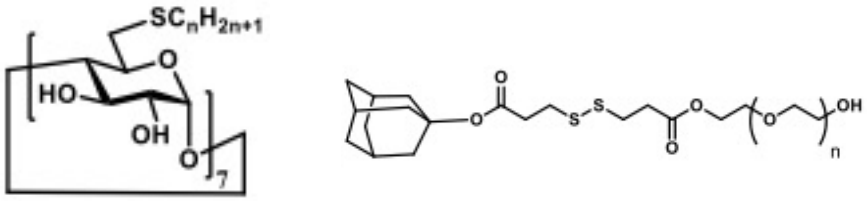


Figure 9. Project goal is to create bilayer vesicles through supramolecular chemistry between hydrophobic modified CD (left) where n=10 or 12 and hydrophilic guest containing a redox sensitive disulfide bond (right).

This method of vesicle formation is advantageous over Ravoo's procedure because it utilizes supramolecular chemistry. Ravoo's modified CD were amphiphilic by themselves, as there was a hydrophobic chain on the primary side and hydrophilic chains on the secondary side. These modifications were enough to drive hydrophobic/hydrophilic interactions to form bilayer membranes. However,

this project uses supramolecular interactions between hydrophobic hosts and hydrophilic guests in order to create these bilayer vesicles. Modification of the secondary side of CD is more difficult and time consuming than only modifying the primary side, so CD used in this project are quicker and easier to synthesize. The guest molecule is needed to form vesicles and are easy to couple several types of end groups to. These guests are also targets for controlled release of cargo, as they can be synthesized with easily breakable bonds that cause the degradation of the bilayer membrane, as with a redox-sensitive disulfide linkage. This schematic can be observed below in Figure 10.

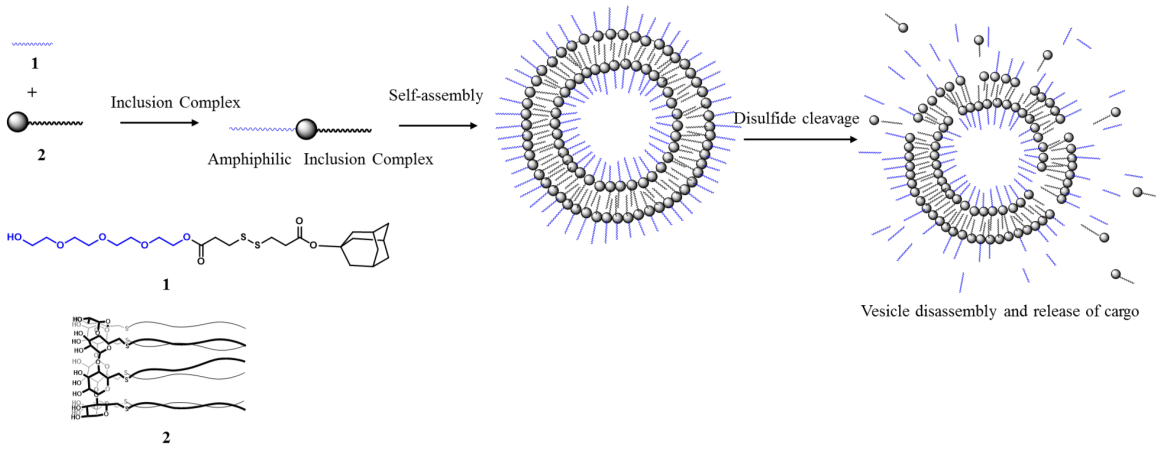


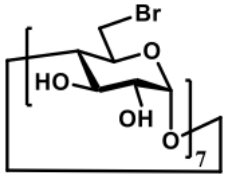
Figure 10. Schematic of vesicle self-assembly and subsequent degradation and release of cargo.

2. EXPERIMENTAL METHODS

2.1 Material Synthesis

All commercial reagents were used as supplied unless otherwise stated. ^{1}H NMR spectra were recorded on a FT-NMR Bruker Avance 300 MHz BioSpin. Vesicles were isolated in Dynamic light scattering experiments performed on a Wyatt Technologies DynaPro NanoStar. Spectrofluorimeter Fluorescence experiments were carried out using a Horiba Fluorolog equipped with an injection port constructed in our lab from black cardboard. Extrusions were performed using a mini-extruder purchased from Avanti Lipids using polycarbonate membranes with 100 nm pore size. Dialysis was carried out using Float-A-Lyzer G2 dialysis devices purchased from Spectrum.

Heptakis(6-bromo)- β-cyclodextrin

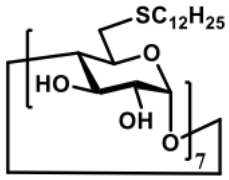


[1]

Stock β–cyclodextrin (β-CD) (4 g, 3.5 mmol) was dried by dissolving in dimethylformamide (DMF) (~30 mL), then placed on rotary evaporation to remove all solvent. Triphenylphosphene (PPh_3) (18.4 g, 70 mmol), dissolved in DMF (~20 mL), and purged under nitrogen three times. Bromine (Br_2) (11.2 g, 70 mmol) was added drop-wise to PPh_3 via syringe and stirred at 60° C for 30 minutes. The dried β-CD in DMF (~20 mL) was added drop-wise. The reaction was stirred overnight

(~18 hours) at 80° C. The reaction mixture was then placed under rotary evaporation to reduce solvent to half volume, then added to ~ 150mL methanol (MeOH). The pH was adjusted to 10-12 with potassium t-butoxide and stirred at room temperature for 30 minutes. The reaction was added to ice water and vacuum filtered over a frit. The precipitate was moved to a Falcon tube and dissolved in MeOH. The sample was centrifuged three times in 5-minute intervals at 7500 RPM. The MeOH was removed, and the precipitate was dissolved in DMF. After rotary evaporation to remove all solvent, the product was dried under high vacuum overnight (~12 hours) to obtain white powder. Figure 11 depicts the NMR results obtained.

Heptakis(6-hexadecylthio)- β-cyclodextrin

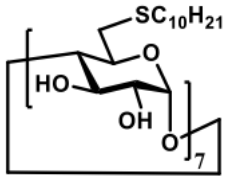


[2]

Bromocyclodextrin (0.315 g, 0.2 mmol), dodecanethiol (0.488 g, 2.8 mmol), and potassium t-butoxide (0.314 g, 2.8 mmol) were added to 50mL RBF with stir bar and purged with nitrogen three times. Anhydrous DMF (~20 mL) was added via syringe to cover the reaction, and stirred overnight (~18 hours) at 80° C. The contents of the RBF were added to excess ice water and vacuum filtered over a frit. The product was added to rapidly stirring MeOH (80 mL) at 50° C for an hour to remove excess thiol. The solution was vacuum filtered again over a frit.

Precipitate was added to a 25mL RBF and dried completely under high vacuum overnight (~12 hours) to obtain fluffy white powder.

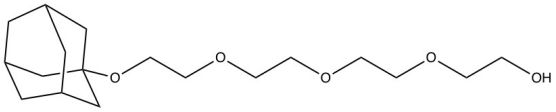
Heptakis(6-decylthio)- β-cyclodextrin



[3]

Bromocyclodextrin (0.35 g, 0.22 mmol), decanethiol (0.542 g, 3.1 mmol), and potassium t-butoxide (0.35 g, 3.1 mmol) were added to 50mL RBF with stir bar and purged with nitrogen three times. Anhydrous DMF (~20 mL) was added via syringe to cover reagents, and the reaction was allowed to stir overnight (~18 hours) at 80° C. The contents of the RBF were then added to excess ice water and vacuum filtered over a frit. The product was added to rapidly stirring MeOH (80 mL) at 50° C for an hour to remove excess thiol. The solution was vacuum filtered again over a frit. Precipitate was added to a 25mL RBF and dried completely under high vacuum overnight (~12 hours) to obtain fluffy white powder.

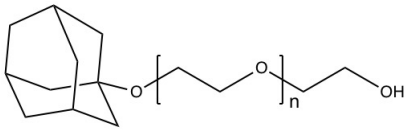
Ad-PEG



[4]

Bromoadamantane (2 g, 9.3 mmol), tetra(ethylene glycol) (TEG) (10.8 g, 55.8 mmol), and triethylamine (2.73 g, 26.97 mmol) were added to 50mL RBF with stir bar. Reaction stirred overnight (~18 hours) at 180° C, then allowed to cool to room temperature. Dichloromethane (DCM) (~30 mL) was added to the room temperature mixture. In a separatory funnel, the solution was washed four times with 2M HCl and once with brine. The organic layer was dried over $MgSO_4$, and the solvent was removed under reduce then placed under rotary evaporation until a thick brown oil remained.

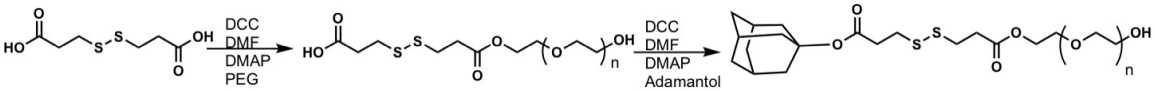
Ad-PEG



[5]

Bromoadamantane (2 g, 9.3 mmol), poly(ethylene glycol) with an average molecular weight of 200 g/mol (PEG) (11.16 g, 55.8 mmol), and triethylamine (2.8 g, 27.9 mmol) were added to 50mL RBF with stir bar. Reaction stirred overnight (~18 hours) at 180° C, then allowed to cool to room temperature. DCM (~30 mL) was added to room temperature mixture. In a separatory funnel, solution was washed four times with 2M HCl and once with brine. Organic layer was further dried over $MgSO_4$, then placed under rotary evaporation until a thick brown oil remained.

Ad-SS-PEG



[6] **[7]**

PEG (3 g, 15 mmol), dithiodipropionoic acid (9.46 g, 45 mmol), and dimethylaminopyridine (DMAP) (0.55 g, 4.5 mmol) were added to 100mL RBF with stir bar and purged with nitrogen three times. Anhydrous DCM was added via syringe to cover reaction (~25mL) and stirred in ice bath until it reached 0° C. Dicyclohexylcarbodiimide (DCC) (3.4 g, 16.5 mmol) was dissolved in 5mL DCM and added to reaction drop-wise via syringe. Reaction was stirred at 0° C for one hour, then warmed to room temperature and stirred overnight (~20 hours). Contents of RBF were added to separatory funnel with deionized water (~25 mL), and DCM was removed. Keeping the aqueous layer, the solution was washed twice with DCM. The aqueous layer was placed under rotary evaporation to remove water. The film was rehydrated with nanopure (Milli-Q, 18 MΩ) water and placed in dialysis membrane. The membrane was placed in a large beaker of nanopure water and gently stirred overnight (~20 hours) at room temperature. The contents of the dialysis membrane were added to small Falcon tubes and frozen under liquid nitrogen. The water was removed from the product **[6]** under a lyophilizer overnight (~18 hours).

PEG-SS **[6]** (from pervious procedure) (0.183 g, 0.466 mmol), Adamantanol (0.71 g, 4.66 mmol) and DMAP (0.028 g, 0.233 mmol) were added to 250 mL RBF with stir bar. The flask was sealed and purged with nitrogen three times. Anhydrous DCM was added via syringe to cover reaction, ~25mL. Reaction was stirred over

ice bath to reach 0° C. DCC (0.29 g, 1.4 mmol) was dissolved in 5mL dry DCM, and then added drop-wise to stirring reaction via syringe. The reaction stirred at 0° C for one hour, then allowed to warm up to room temperature and stirred overnight (~20 hours). Contents were added to separatory funnel with ~25 mL DI water. After removing the DCM, the aqueous layer was then washed twice with DCM. The aqueous layer was placed under rotary evaporation to remove water. To rehydrate film, 7mL nanopure water was added, and then moved to dialysis membrane. The membrane was stirred in large beaker of nanopure water overnight (~20 hours) at room temperature. The contents of the dialysis membrane were added to small Falcon tubes and frozen under liquid nitrogen. The water was removed from the product under a lyophilizer overnight (~18 hours).

2.2 Vesicle Preparation

2.2.1 Vesicles for DLS Studies

The general procedure for vesicle formation was consistent throughout all the trials. However, there were certain variables that were altered from trial to trial in order to optimize the vesicle formation. The general procedure started with adding modified cyclodextrin, [**2**] or [**3**], to 25mL RBF, with guest molecule if applicable, and dissolved in ~ 1mL chloroform, then placed under rotary evaporation to remove all solvent and leave a thin film. The film was then hydrated with 1 mL Tris buffer (10 mM, pH 7.4) for one hour. The vesicle formation procedure was either by sonication or extrusion. Early samples were sonicated at room temperature for one hour, then filtered through a 0.45 μm PTFE syringe filter.

Sample was then added to DLS cuvette and ~1 mL Tris buffer was added to completely fill the cuvette. Samples that used extrusion as vesicle formation procedure first employed freeze-thaw cycles to make vesicles at their lowest energy state. Vesicle samples were frozen in dry ice in acetone and thawed in a 60° C water bath a total of 5 times. The sample was then filtered through a 0.45 μm PTFE syringe filter, then extruded through a 100 nm porous membrane a total of 5 times (extruder pictured in Figure 11). Sample was then moved to a DLS cuvette and ~1 mL Tris buffer added to completely fill the cuvette. Several variables were tested in order to optimize the vesicle formation procedure to determine the most efficient way. One such variable was dissolving the CD (and guest, if applicable) in chloroform at an elevated temperature of 50° C versus room temperature. Another variable explored was the film hydration temperature, either 50° C or room temperature. Finally, the ratio of guest to host equivalents was also changed.

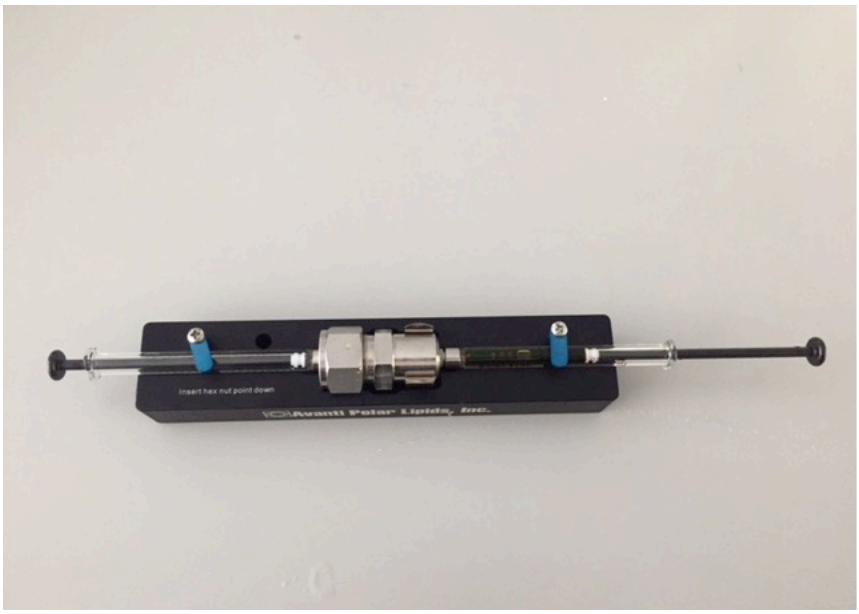


Figure 11. Extruder with membrane of pore size 100 nm used for vesicle isolation.

2.2.2 Vesicles for Fluorescence Studies

To prepare fluorescent studies the same procedure was followed for the vesicle preparation above using a CF buffer (10 mM CF, 10 mM Tris, pH 7.4). After extrusion, the sample was passed through Sephadex gel permeation chromatography in order to separate the dye-loaded vesicles from the free CF molecules in buffer (Figure 12). The sample separated into three different colored bands: brown, yellow and orange. The brown band was large aggregates of excess Ad-PEG. The yellow band was determined to be the free CF dye in buffer, as the dye is orange at self-quenching concentrations. Therefore, the orange band was used for fluorescence studies, as the dye encapsulated by vesicles were at self-quenching concentrations.

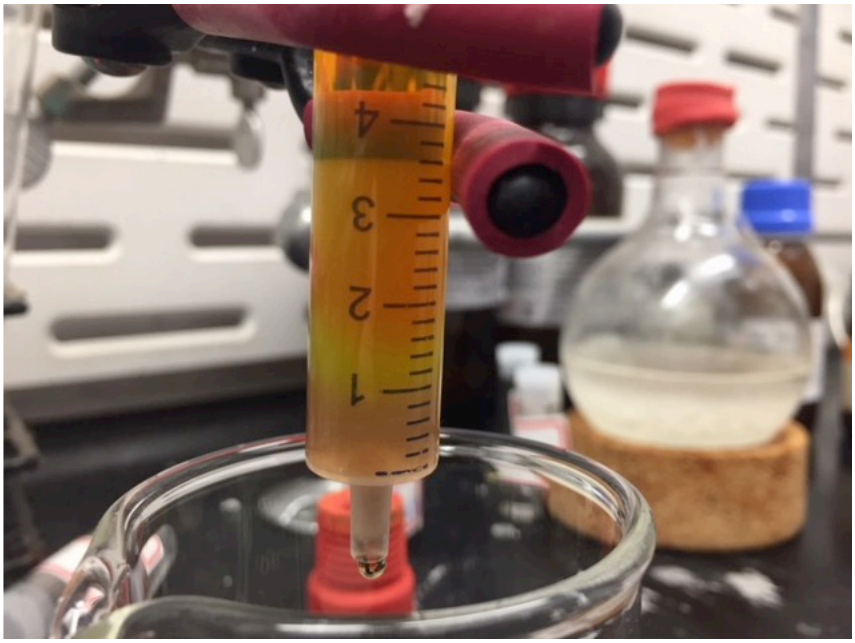


Figure 12. Sephadex column separating particle sizes for fluorescence studies. Excess Ad-PEG measures at 0-1.0 mL (brown), free CF dye at 1.0-1.6 mL (yellow), and CF-containing vesicles at 1.6+ mL (orange).

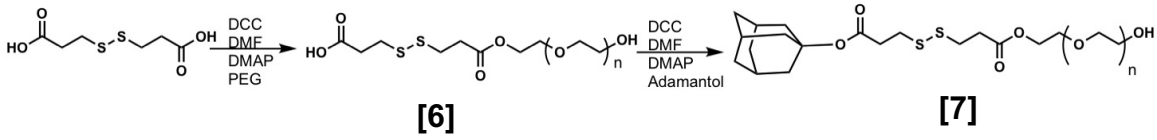
2.3 Fluorimeter Procedure

In glass cuvette, 2000 µL of Tris buffer solution and 25 µL of vesicle-containing sample were added with stir bar. Cuvette was placed in fluorimeter and the fluorescence was observed at λ_{em} 517 nm and λ_{ex} 492 nm. The total run time was 120 seconds, data taken at 0.1-second intervals. At t = 40 seconds, 20 µL of Triton X was added to the cuvette to lyse the vesicles.

3. RESULTS AND DISCUSSION

3.1 Materials Synthesis

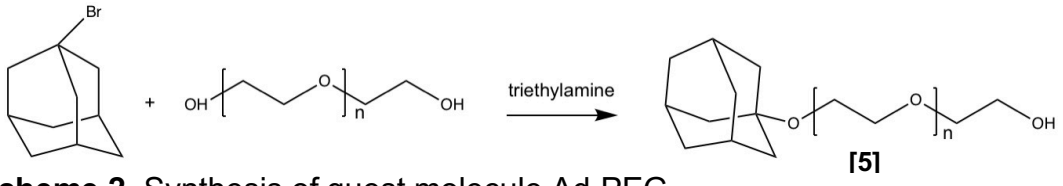
The main goal of this project was to synthesize a guest molecule that contains a disulfide bond in order to form a nano-assembly that is degraded when the redox sensitive disulfide bond is cleaved. The desired guest would contain an adamantane one on end for inclusion into the CD and a PEG chain on the other to provide a hydrophilic region to create an amphiphilic nano-assembly. The disulfide bond would be in-between these two to form Ad-SS-PEG, as depicted in Scheme 1 below **[7]**. The process first couples a PEG chain to dithiodipropionoic acid, which contains a disulfide bond between carboxylic acid end groups. Using dicyclohexylcarbodiimide (DCC) and dimethylaminopyridine (DMAP) as coupling agents, the alcohol of the PEG chain and the carboxylic acid interact in order to tack the PEG chain on to the disulfide bond to create **[6]**. The second step of the process uses the same coupling agents and adamantanol, to couple the adamantane group to the other end of the disulfide bond.



[6] **[7]**

Scheme 1. Synthesis of guest molecule SS-PEG and Ad-SS-PEG.

Cleavage of this disulfide bond causes the guest molecule to degrade, which in turn breaks apart the nanoparticle complex. The cleavage removes the hydrophilic PEG chain from the guest molecule, and the remaining parts of the assembly are not amphiphilic enough to maintain the bilayer structure. Breaking apart the vesicle membrane releases the encapsulated cargo inside, thus successfully delivering small molecules to a targeted location. The disulfide linkage in the guest molecule is readily cleaved by glutathione (GSH), which is found in higher concentration inside cells compared to extracellular environments. This product is currently still a work in progress, as NMR results do not show the final product and no vesicles were formed. Further work for this project should include using other coupling agents to synthesize this product, as all attempts using DCC have been unsuccessful.



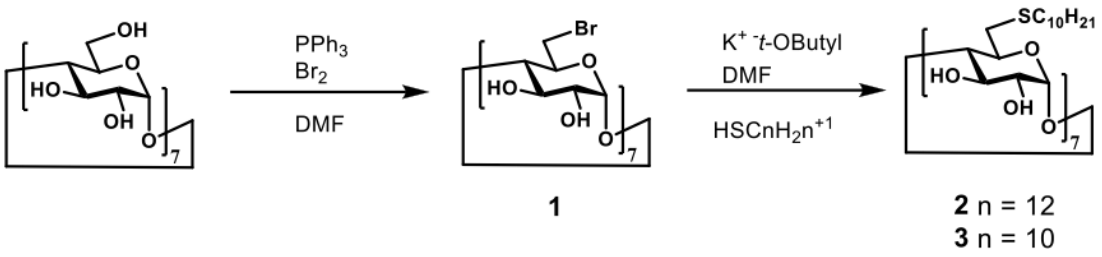
[5]

Scheme 2. Synthesis of guest molecule Ad-PEG.

To determine optimal conditions in which to form vesicles, a different adamantane guest molecule were first synthesized **[5]**. The initial guest contained only adamantane attached to a PEG chain where n = 5, providing the hydrophilic

portion in order to from an amphiphile when complexed with thioalkylated CD. Adamantane derived guest molecule Ad-PEG synthesis, pictured in Scheme 2 above, was performed according to literature, which was simply heating all reagents overnight (~18 hours) at 180° C.[21] Initially, a heating mantel was used, but the fluctuating heat was not steady enough for the reaction to run overnight, and no product was formed. The second attempt was completed with a more stable oil bath, which was successful and produced higher yield.

The synthesis of both bromocyclodextrin and the alkylthiol-modified CDs followed procedures established in the lab, as seen in Scheme 3 below. While the synthesis methods were reliable, several attempts at the modified CDs were necessary before a high enough yield was collected. To create bromocyclodextrin, bromine (Br_2) and stock CD in the presence of triphenylphosphine were reacted used to replace a hydroxyl group on the primary side of the CD with bromine to create **[1]**. After several unsuccessful attempts at further modifying CDs to contain a hydrophobic thioalkyl group, it was determined that the brominated CD needed to be dried under high vacuum overnight (~12 hours) to remove all excess water, as it was preventing the thioalkyl group from being able to bind to the CD. Once the bromo-CD was dried, thioalkylated CDs were successfully synthesized (**[2]** and **[3])** under basic conditions in an S_N2 reaction with **[1]**. All modified CDs were isolated after synthesis by filtering through a frit and collecting the solid precipitate. Products **[2]** and **[3]** were also dried under vacuum overnight to remove all excess water to prevent interference in vesicle formation.



Scheme 3. Synthesis of first brominated cyclodextrin (center) and hydrophobe-containing cyclodextrin (right).

Products were confirmed by and compared to reported data in literature. Figure 13 below depicts the NMR data for compound **[2]**. Important peaks are labelled within the NMR spectrum to show that there are specific structures present within the compound. The sugar protons are the bulk of the cyclodextrin. The S-CH_2- peak at 3.0 ppm shows that the thioalkyl is present, and that the hydrophobic modification was successful. The one doublet for the H-1 proton indicates complete and symmetrical substitution of the thioalkyl chain. If there were substitutions on only 6 positions of the CD then there would be 7 different doublets for the H-1 proton. The triplet at 2.8 ppm shows free thiol within the sample, meaning stirring the compound in hot methanol did not remove all the unreacted thiol from the product.

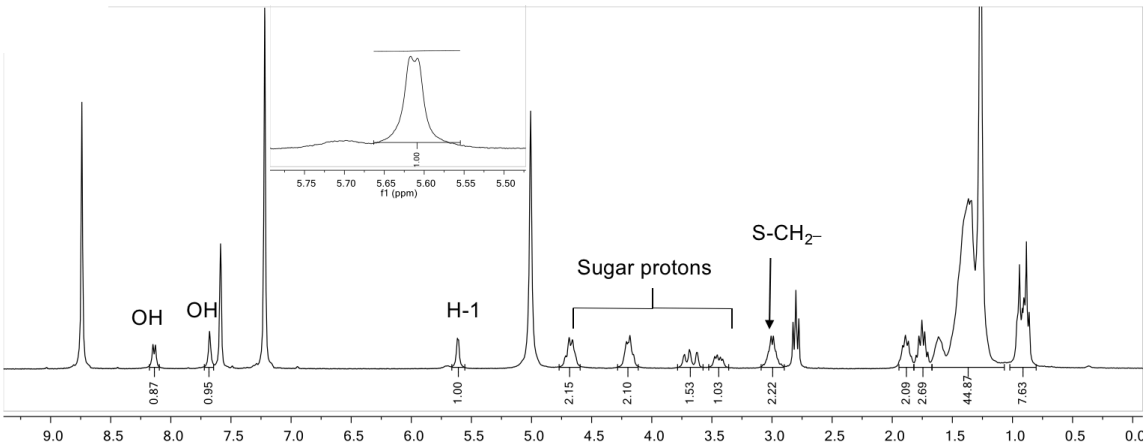


Figure 13. Nuclear Magnetic Resonance for [2].

Figure 14 below depicts the NMR results for **[1]**. The peaks observed corresponded with the peaks described in literature references, so **[1]** was used to further synthesize CD with a hydrophobic thioalkyl chain, either **[2]** or **[3]**.

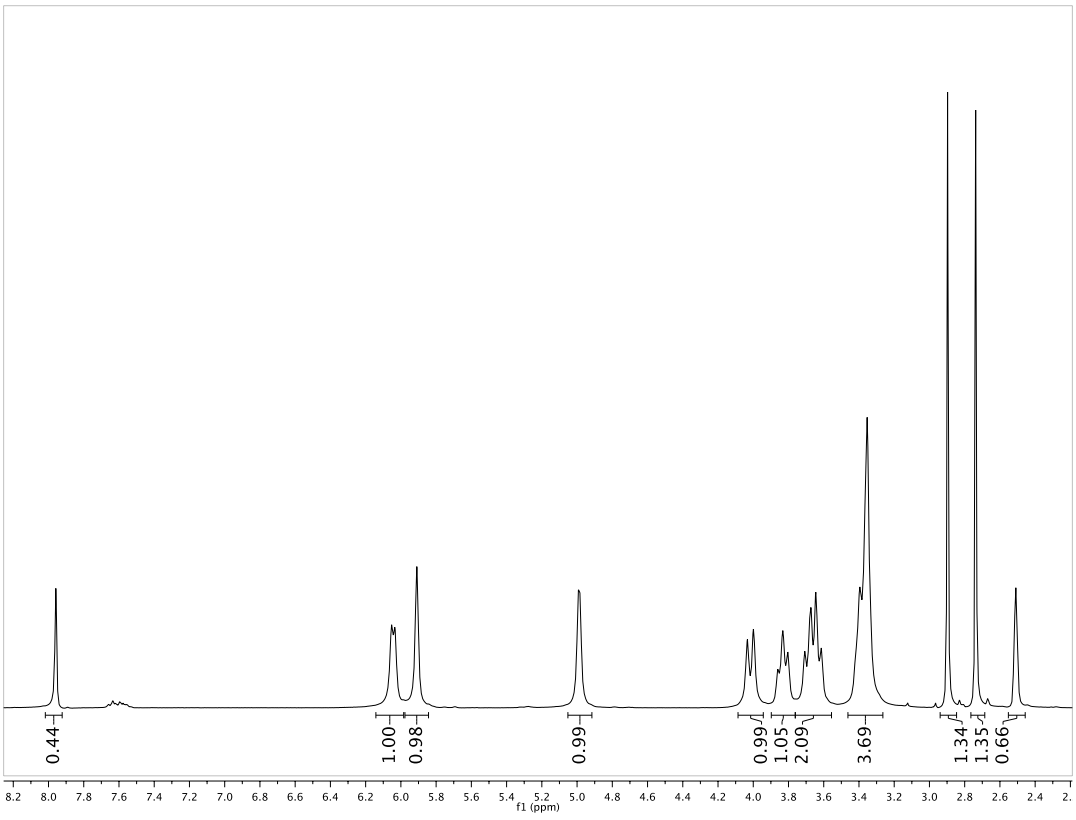


Figure 14. Nuclear Magnetic Resonance for [1].

The guest molecule Ad-PEG was also observed under NMR to determine the success of the synthesis compared to literature. Figure 15 below shows the results. The peaks match with the NMR results presented in the literature references.

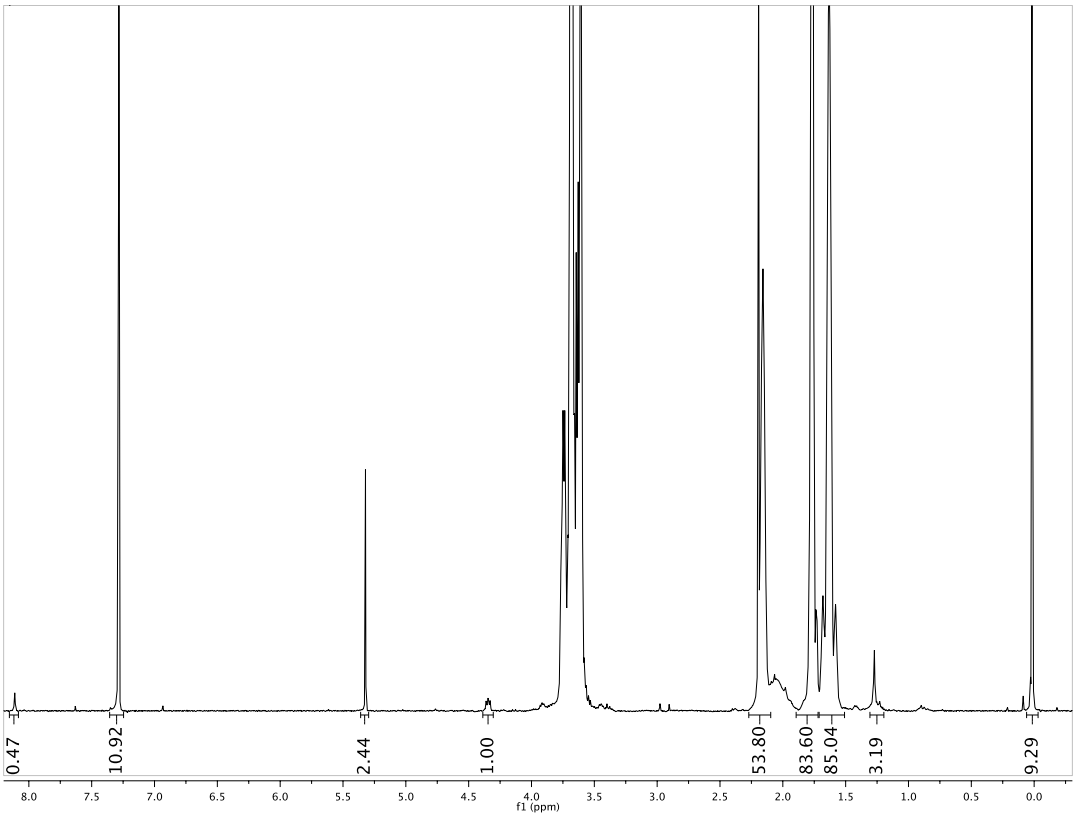


Figure 15. Nuclear Magnetic Resonance for [5].

3.2 Vesicle Preparation Optimization and Particle Size Distribution

The initial attempts to form vesicles closely followed the procedures outlined previously reported, which was as follows: dissolving CD in chloroform and rotary-evaporating to create a thin film, then hydrating the film with Tris buffer for an hour

and sonicating for an hour.[17] Initial DLS measurements showed promising results, so the same procedure was used to attempt CF dye encapsulation. Fluorimeter results did not show any successful dye loading in vesicles, as addition of lysing agent did not cause an increase in fluorescence which would indicate release of trapped dye within the vesicles. Literature review revealed the wrong type of particle size distribution was being observed. Using the correct style of distribution curve, it was discovered that there were no particles present that fell within the literature-accepted size range of vesicles. From there, several variables were tested in order to successfully prepare vesicles.

Table 1 Vesicle Procedure Optimization

Trial	CD	Guest	Ratio	Isolation	Particle Radius (nm)
2	C12	Ad-TEG	1:1	Sonication	4 (62 %), 114 (11%), 711 (28%)
3	C12	Ad-PEG	1:1	Sonication	0.6 (97%)
4[a]	C12	Ad-PEG	1:1	Extrusion	2 (93%), 1306 (4%)
7	C12	Ad-PEG	1:1	Extrusion	3 (62%), 9188 (32%)
8	C12	Ad-PEG	2:1	Extrusion	3 (77%), 11 (8%), 260 (9%)
9[b]	C12	Ad-PEG	10:1	Extrusion	n/a
10	C12	Ad-PEG	5:1	Extrusion	9 (60%), 82 (29%), 317 (11%)
11[c]	C12	Ad-PEG	1:1	Extrusion	3 (85%), 23 (8%), 58 (4%)
12	C12	Ad-PEG	10:1	Extrusion	17(44%), 76 (56%)
14	C10	Ad-PEG	10:1	Extrusion	2 (98%)

Footnote: [a]: CD was dried under high vacuum to remove excess water. [b]: no results available because solution was too chunky to finish extrusion. [c]: film hydration heated to 50° C to improve solution viscosity.

Table 1 above lists all the trials of vesicle preparation. The two methods of sonication and extrusion were tested, as cited in literature.[17] The first few trials used modified CD that had not been completely dried under hi-vac. There was excess water in the CD that interfered with the complete dissolution of product in chloroform during film formation. After excess water was removed starting at trial 4 by placing CD under high vacuum overnight (~12 hours), the CD dissolved more readily. The first few trials of using a guest molecule to help drive bilayer formation used compound **[4]** as the guest. After unsuccessful formation, it was hypothesized that the tetra(ethylene glycol) was too short. A major factor in self-assembly of amphiphiles in aqueous solution is the volume ratio between the hydrophilic and hydrophobic regions of the amphiphilic. We hypothesized that the tetra(ethylene glycol) hydrophilic chain was not long enough to achieve the need balance for self-assembly into vesicles. To remedy this, a new adamantane derivative was synthesized **[5]**. A PEG with an average molecular weight of 200 g/mol was used instead of the shorter TEG chain. Once a working Ad guest molecule was discovered, the next variable to alter was the guest to host ratio. So far, CD and Ad-PEG were added in molar equivalents. It was hypothesized that by increasing the amount of guest molecule would increase the drive to vesicle formation. Increasing to a ratio of 2:1 guest:host did not improve results, so the ratio was increased to 10:1. This trial (9) was particularly difficult to perform due to the high levels of Ad-PEG. The high viscosity of the guest molecule made filtering, extruding, and separating incredibly difficult and slow, and no DLS results were collected. The ratio was then reduced to 5:1, which was not only slightly easier to

prepare than 10:1, but also showed more promising results than the 2:1. At this point there was an idea to heat the film hydration process, starting with trial 11. The RBF was placed in a 50° C oil bath while the buffer hydrated the film. This made the rest of the vesicle formation process much easier, even the 10:1 ratio trials. Another factor that improved the vesicle formation was using heat to help dissolve the CD (and guest molecule, if applicable) in the chloroform before film formation (see Trial 6). This helped create more evenly distributed films. Breaking apart aggregates of CD improved the ability to properly form vesicles. One other variable that was tested was completely drying the film under high vacuum after rotary evaporation to remove all excess chloroform. This would prevent interference of the chloroform during film hydration. No positive change was observed in the DLS results after trying this, so drying the film under vacuum was discontinued.

Table 2. Vesicle Preparation Negative Controls

Trial	CD	Guest	Ratio	Isolation	Particle Radius (nm)
1	C12	None	n/a	Sonication	4 (97%)
5	C10	None	n/a	Extrusion	0.6 (100%)
6	C12	None	n/a	Extrusion	2 (84%), 1603 (11%)
13	None	Ad-PEG	10:0	Extrusion	5 (36%), 133 (51%), 752 (11%)
17	C12	PEG	10:1	Extrusion	2 (99%)

Several negative controls were employed to ensure that positive results were truly due to the controlled variables. Table 2 above summarizes the trials that were used as negative controls. Trials 1, 5, and 6 show that a guest molecule is needed in order to form vesicles, as modified CD does not form 3D complexes by

itself. Trial 13 shows that the guest molecule Ad-PEG does not form these complexes by itself either. To determine the necessity of adamantane in forming the inclusion complex, vesicles were attempted using C12 **[2]** and poly(ethylene glycol) with an average molecular weight of 200 g/mol. This PEG is the same compound used to synthesize the Ad-PEG guest **[5]** that successfully formed vesicles. Using just PEG and modified CD, no vesicles were observed in DLS measurements, proving the necessity of adamantane in forming guest-host amphiphilic inclusion complexes.

The size and quantity of particles present in each sample of vesicle preparation were observed via dynamic light scattering (DLS). Initially, the intensity distribution was used to characterize the sample. Intensity distributions show the percentages of each observed diameter in terms of how intense the reflected light is.[22] The larger particle sizes reflect more light than the smaller particles, and therefore result in a much higher percentage. Even though there could be a smaller population of these large particles, they still report a larger intensity distribution. This was the incorrect type of distribution to observe because it does not accurately portray the populations of each measured diameter. The first couple of vesicle preparation methods were characterized using this type of distribution. This mistake was realized when vesicle preparation procedures thought to have been successful did not show signs of fluorescent dye encapsulation (further discussed below in Section 3.3). Upon further literature review, it was determined that the mass distribution was the accurate measurements to observe presence of vesicles formed.[17,20,22] The mass distribution displays the amount of each diameter

category as a percentage of the total mass of particles observed. This is the correct distribution to observe because the goal was to form a large population of vesicles and separate out both aggregates and smaller free molecules. Ravoo reported vesicles formed under sonication averaged 80-100 nm in diameter, while extruded vesicles were larger, at 140-160 nm.[17] These values were used to determine whether vesicles were properly prepared.

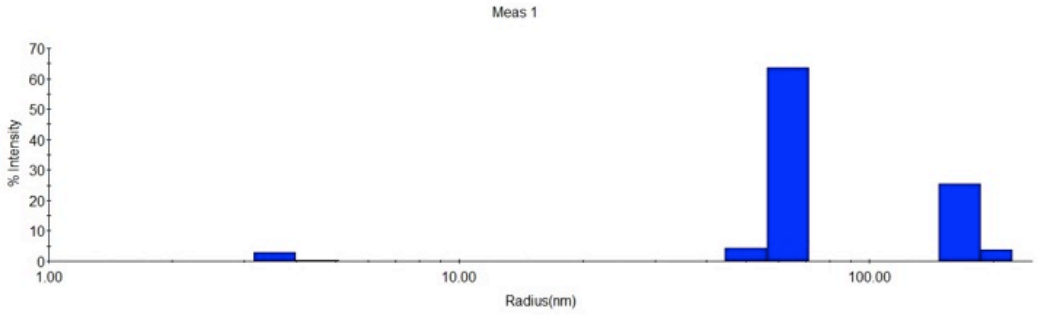


Figure 16. Intensity Distribution of Unsuccessful Vesicles from Trial 1 using undried C12 and Tris buffer.

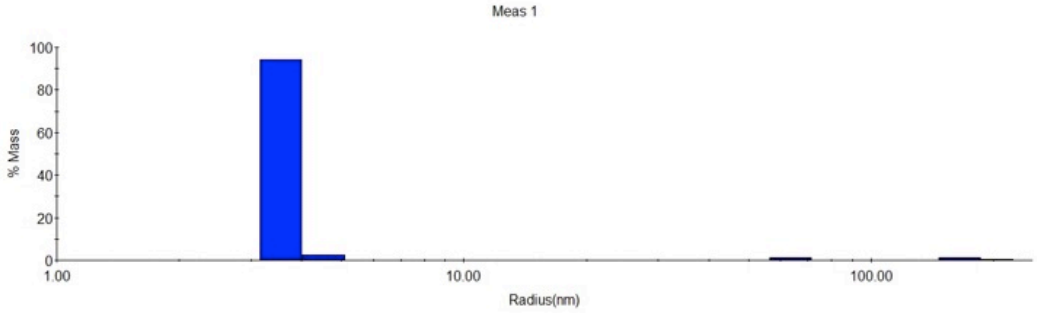


Figure 17. Mass Distribution of Unsuccessful Vesicles from Trial 1 using undried C12 and Tris buffer.

Figure 16 and Figure 17 show the difference between the intensity and the mass distributions of one of the initial vesicle preparation samples. The intensity distribution shows a very higher percentage of particles at diameter 62.6 nm and 171.5 nm, which was initially taken to mean the majority of the particles present in the sample were of those sizes. Upon reviewing literature, the mass intensity was examined. Even though the intensity percentage was so high for diameters 62.6 nm and 171.5 nm, the mass percentage was zero, meaning no vesicles were formed. Realization of this lead to further modifications of the vesicle preparation methods.

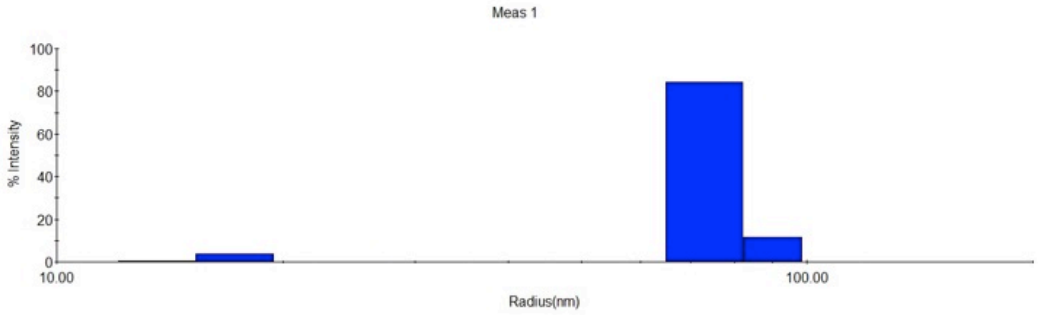


Figure 18. Intensity Distribution of Successful Vesicles from Trial 12 using C12 and Ad-PEG in a 1:10 ratio.

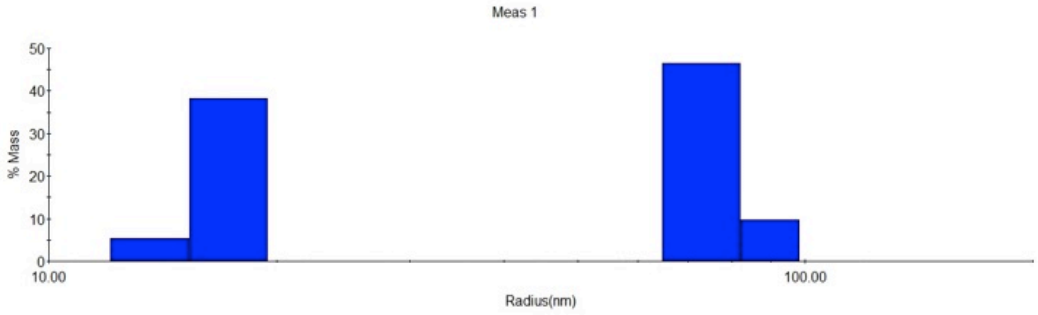


Figure 19. Mass Distribution of Successful Vesicles from Trial 12 using C12 and Ad-PEG in a 1:10 ratio.

Figure 18 and Figure 19 displays the DLS measurements for successful vesicle preparation. The intensity distribution (Figure 21) shows almost all of the particles present in the sample had a diameter of 76.4 nm, similar to the intensity distribution of unsuccessful vesicles. Looking at the mass distribution (Figure 22), the percentage for the proper vesicle size range according to literature is significantly higher than the samples from initial preparation methods. The mass distribution shows that majority of the total mass (56.4%) was from particles of 76.4 nm, and less than half was from particles of 16.8 nm. It is likely that single-layered micelles were formed in addition to the bilayer vesicles, which would be around the size range of 17 nm in radius. The intensity distributions of the successful and the unsuccessful vesicles both showed a high percentage for particles around 80 nm, which is the correct size according to literature. However, the mass distribution differences between the two trials show that almost no particles of the right vesicle

size are present in Figure 20, while the successful vesicle trial in Figure 22 had a much larger mass percentage, indicating the presence of vesicles.

DLS data was not available for vesicles prepared with the CF buffer due to the fluorescent molecules. The fluorescence interrupted and interfered with the lasers in the DLS instrument, so no accurate data on particle size distribution was acquired.

3.3 Encapsulation

Based on promising DLS measurements, a vesicle preparation method was executed using CF loaded dye in place of the Tris buffer to test for encapsulation. If DLS showed particles falling within the range of reported vesicle size, the same procedure was used to load vesicles with fluorescent dye. Carboxyfluorescein dye was added to Tris buffer at self-quenching concentrations, so the molecules do not fluoresce. CF is a bright orange powder and turns the buffer a bright orange as well. Dilution of this buffer solution changed the color to a luminescent yellow-green. The vesicle sample separated into two bands during the Sephadex column, a bright yellow band and an orange band. This column separates large molecules from small ones, specifically isolating dye-loaded vesicles from free CF in the buffer solution. It was decided that the orange band contained the vesicles, as the encapsulated dye was at self-quenching concentrations.

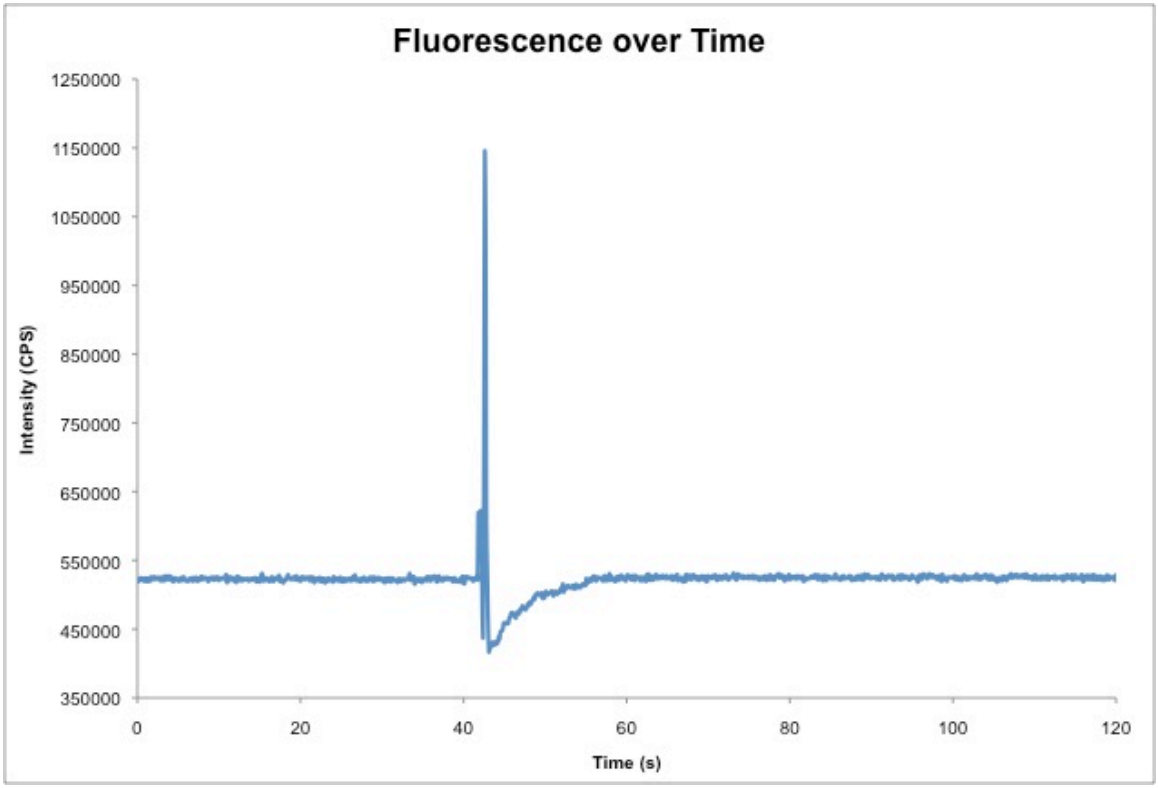


Figure 20. Fluorimeter Results of Unsuccessful Encapsulation using the procedure from Trial 1.

Figure 20 is a fluorimeter measurement of unsuccessful dye encapsulation using the vesicle preparation procedure from Trial 1. The cuvette was equipped with a stir bar and stirred throughout the duration of the experiment. The baseline fluorescence measurement was established from t = 0 – 40s to account for any free CF in the buffer or diffusion from the vesicle. At 40 seconds Triton X was injected. To minimize light pollution as much as possible, a port with a small hole just large enough for a pipette tip was constructed from black cardboard. A thick piece of black cloth was used to cover the port hole until it was time to add the Triton. While these measures helped reduce light pollution and noise in

fluorescence measurements, completely eradicating background light was not possible, and can be seen in measurements at the time when Triton was added. The fluorescence experienced a sharp peak when the port cover allowed light into the fluorimeter, but the fluorescence returned to the baseline once the cover was replaced. This indicates the sample solution did not successfully encapsulate any dye inside the vesicles. Addition of the lysing agent Triton X did not cause degradation of vesicles, which would have released any trapped CF dye. The CF dye present in the sample was already dilute enough to overcome self-quenching, so it was at peak fluorescence before the addition of the lysing agent.

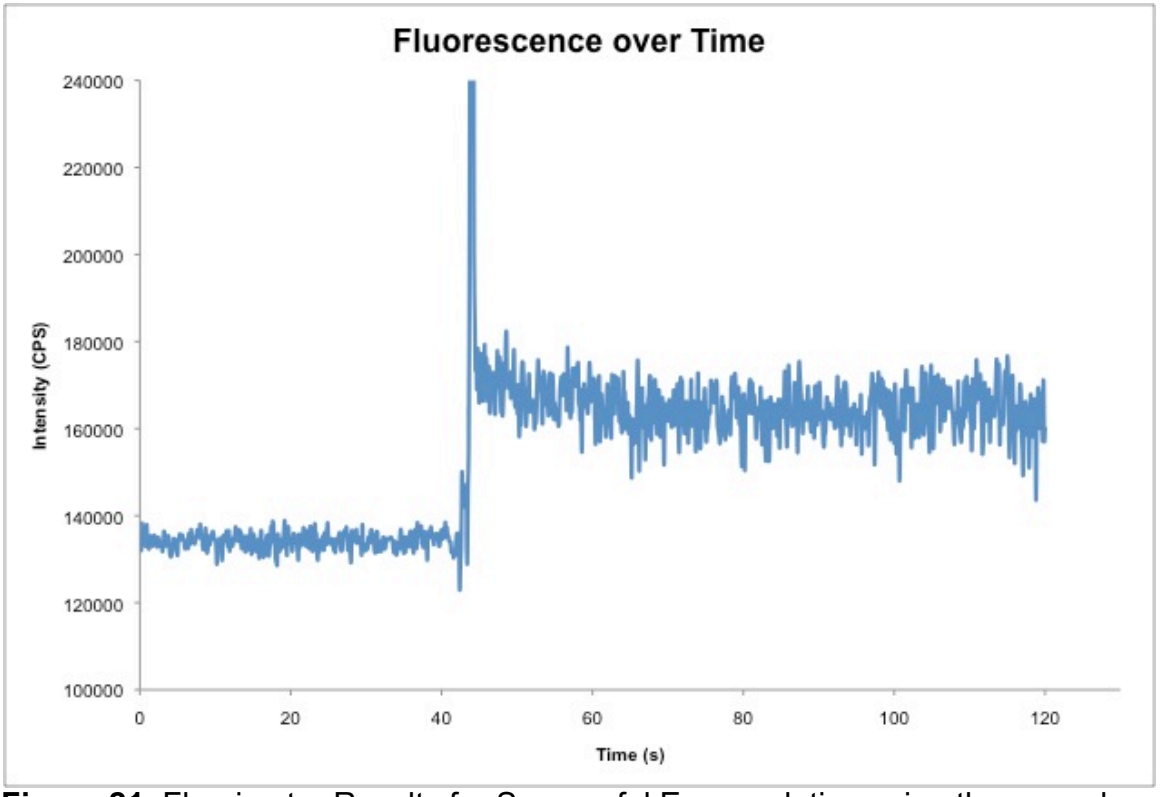


Figure 21. Fluorimeter Results for Successful Encapsulation using the procedure from Trial 12.

Figure 21 shows the change in fluorescence of a successful encapsulation from using the procedure outlined in Trial 12. After addition of the lysing agent at 40 seconds, the fluorescence experiences a sharp peak and levels off at around 180000 CPS. Addition of Triton X causes the bilayer membrane to fall apart, thus releasing the fluorescent cargo into the solution. The baseline fluorescence of around 140000 CPS is the self-quenching emissions, and the increased measurement of ~1.3 times the baseline is when the CF molecules are dilute enough in solution to fluoresce freely. The sharp increase in fluorescence should ideally be smooth, yet it has a lot of noise. This can be caused by light pollution as the port cover is lifted to administer the Triton X. Overhead lights from the room and natural light from the windows could have affected the fluorescence reading while the lid was partially lifted to add the lysing agent to the sample. Several attempts at smoothing the process were unsuccessful, and the rough spike in measurements was present in all trials. This fluorescence study indicates that the vesicle preparation procedure detailed in Trial 12 was truly successful in not only vesicle formation, but that encapsulation was achieved as well with this procedure.

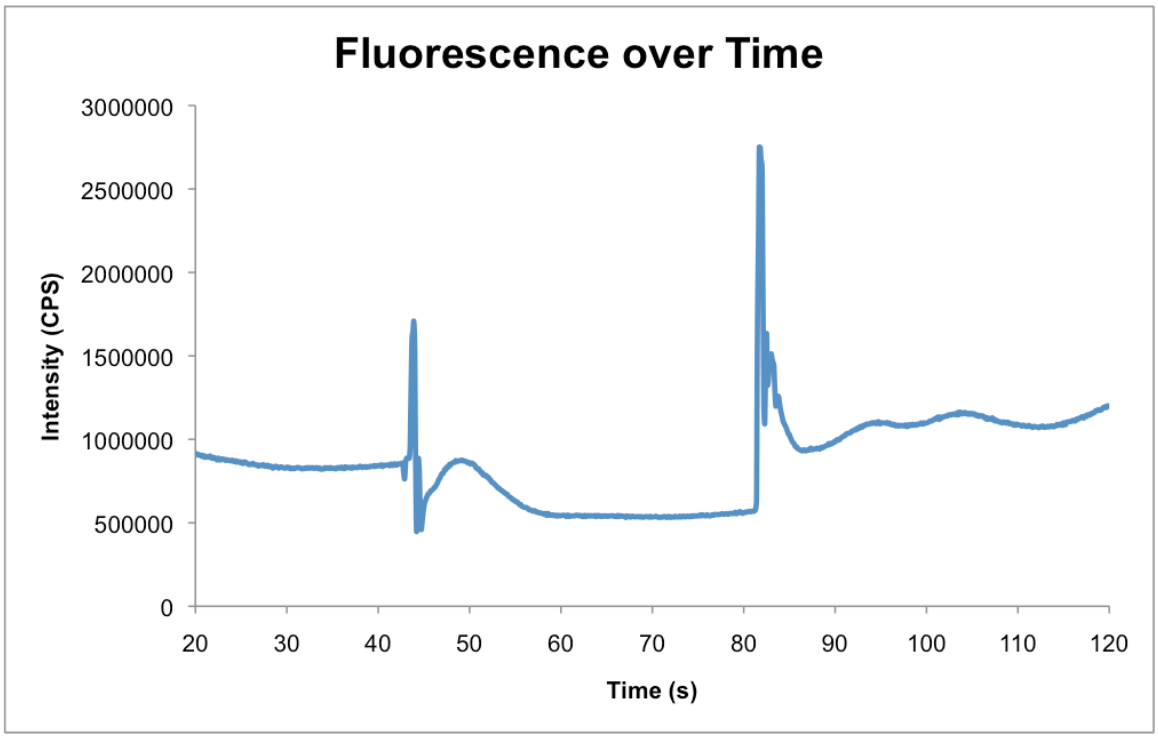


Figure 22. Fluorimeter Data for C12 and Ad-PEG vesicles treated with GSH buffer (t = 40s) and Triton X buffer (t = 80s).

Figure 22 above depicts the fluorimeters results from a trial of comparing the lysing differences between GSH buffer and Triton X buffer. The vesicles were composed of C12 and Ad-PEG, as outlined in Trial 12. This experiment was performed to determine the effectiveness of GSH buffer on lysing vesicles that have no disulfide bond. The GSH buffer was injected at t = 40 s, and it can be observed that the fluorescence decreased from the baseline fluorescence. This decrease in fluorescence shows that the glutathione has no effect on the stability of the vesicles and does not cause degradation of the bilayer membrane. The vesicles remain intact in the presence of GSH, and the sample only becomes

diluted with the addition of the glutathione buffer. The concentration of GSH in the buffer is in the range of intracellular concentrations (6 mM).[18] This trial is to use as a negative control to compare to vesicles made with Ad-SS-PEG to show that GSH is necessary to break the disulfide bonds and cause degradation of the vesicle membrane, releasing the entrapped cargo. From this trial, it is seen that GSH has no effect on breaking vesicles that do not contain disulfide bonds. At t = 80 s, the Triton X buffer was injected, to show that there were vesicles in the sample that successfully encapsulated the CF dye. After the Triton X buffer was introduced, the fluorescence of the sample increased from the diluted levels after GSH buffer was added.

4. CONCLUDING REMARKS

While the ultimate goal of developing a supramolecular complex composed of hydrophobic-modified cyclodextrin and an Ad-derived guest molecule containing a cleavable disulfide bond was not achieved in this project, there were several smaller successful steps that set a solid foundation for future progress. The hydrophobic CDs were successfully synthesized, as seen in both the C10 and C12 compounds. An intermediate guest molecule, Ad-PEG, was also successfully synthesized and used to determine the optimal vesicle formation procedure. Encapsulation was also successfully proven by loading fluorescent dye into the vesicles and lysing them to measure change in fluorescent intensity. Several negative controls were used to show the credibility of the successful experiments.

Modifying the CDs in a way that makes addition of a guest molecule necessary for vesicle formation is important for future drug delivery applications. The inability to prepare vesicles without the guest molecule is important for cargo release purposes. Targeted delivery can be achieved by structuring the degradation of the guest molecule under specific conditions that would cause the vesicle to fall apart. Breaking apart the bilayer membrane then releases cargo. Previous work has modified cyclodextrins to form vesicles from their own hydrophobic and hydrophilic interactions, with no guest molecule needed.

There are several methods to continue on this project. The most important one is to successfully synthesize the disulfide containing adamantane guest molecule. A coupling agent other than DCC should be found, as all attempts using it were unsuccessful. Another one is observing the cellular uptake of these supramolecular nanoparticles. By using live cell cultures, transport of these vesicles can be characterized and optimized. As the main purpose of creating these vesicles is to deliver cargo into live cells, this future direction would determine the overall success of the project. Another possibility is the use of scanning electron microscopy and/or transmission electron microscopy to view the guest-host complexes. Visual characterization of the vesicles could provide important data on how well the guest molecules are included into the hydrophobic regions of the CDs. Use of a confocal microscope can provide important characterization of the diffusion properties. This is important to be able to predict accurate dosing and time-release of the encapsulated cargo. Drug-loaded vesicles can be used in several administrative routes, such as orally, parenteral injections,

or transdermally. Being able to observe the diffusion characteristics through Brownian motion could be used to predict the behavior of these vesicles under real physiological settings. Since successful encapsulation of hydrophilic molecules was achieved, it would be interesting to attempt encapsulation of hydrophobic molecules. A large majority of pharmaceutical products are hydrophobic, so being able to load them into the membrane of these vesicles could potentially improve bioavailability of these drugs.

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